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In re application of:

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Examiner: John Ulm

For: METHOD OF SEARCHING FOR PHYSIOLOGICALLY ACTIVE SUBSTANCES AND PROCESS FOR PRODUCING THE SAME

Attorney Docket No.: IKU 0102 PUSA

TRANSMITTAL OF SUBSTITUTE SPECIFICATION UNDER 37 C.F.R. § 1.125

Commissioner for Patents
United States Patent and Trademark Office
Washington, D.C. 20231

Sir:

Enclosed is a substitute specification for the originally filed specification in this application. Kindly delete the entire previous specification and insert the following replacement specification:

Specification

Field of the Invention:

The present invention relates to a method for identifying new physiologically active peptides and producing said peptides.

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8

I hereby certify that this paper, including all enclosures referred to herein, is being deposited with the United States Postal Service as first-class mail, postage pre-paid, in an envelope addressed to: [redacted], Commissioner for Patents, United States Patent and Trademark Office, Washington, D.C. 20231 on:

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Background Art

Physiologically active substances of unknown types have been discovered previously by analyzing substances present in body fluid or tissues, identifying and separating these substances, and then determining their physiological activities.

Many physiologically active substances are often present at very low concentrations, thus making them difficult to identify. Furthermore, because living organisms are comprised of numerous physiologically activities, it is difficult to identify the physiologically activities of a newly isolated substance. Thus, the prior art methods render it difficult to identify and isolate new physiologically active substances.

Summary of the Invention

We have found that it is possible to isolate new physiological active peptides with greater efficiency and predictability than the methods of the prior art.

In particular, according to the present invention, a method has been found for identifying physiologically active peptides. Some receptors, although encoded by the same gene, have one or more variants in size because of alternative splicing of the gene or post-translational modification. We have found that the domain encompassed by the amino acid sequence of the missing portion of the smaller receptor has physiological activity if one of two conditions are met: 1) either a substance or cell exists in vivo which is functionally antagonistic to the ligand of the receptor, or 2) a substance or cell exists in vivo which is functionally antagonistic to cells which express the receptor.

For example, calcitonin binds to calcitonin receptors present on osteoclasts to suppress the deossification function of osteoclasts. Osteoblasts function is a manner antagonistic to the deossification function of osteoclasts. It has been reported that there are one or more variants of the calcitonin receptor and although these variants are of different sizes,

they are the product of the same gene. Thus, the difference between the two variants of the receptor is that one variant contains a sequence of amino acids that is not present in the smaller variant because of alternative splicing or some post-translational modification. We predicted that a peptide representing the sequence of amino acids which are present in the larger variant of the calcitonin receptor, but not the smaller variant, would have physiological effects on osteoblasts, which are antagonistic to the function of osteoclasts. After chemically synthesizing the peptide which encompassed these amino acids, we demonstrated that this peptide promotes osteogenesis, a function antagonistic to the deossification caused by osteoclasts. We have also demonstrated that this peptide reacts by binding to receptors which are present on the osteoblasts. These results confirmed our prediction and provide the basis for the present invention.

In other words, the present invention provides a method for identifying physiologically active peptides, the method comprising the steps of (1) identifying amino acid sequences of receptors having one or more variants in size, the receptors being receptive of an identical ligand and being products of the same gene, wherein there is a substance or cell present in vivo having a functional antagonism against the ligand for the receptor or against a cell which expresses the receptor of the ligand, and (2) identifying which domain in the larger receptor is missing from the smaller receptor. The present invention also provides a method for producing physiologically active peptides, the method comprising the step of producing the sequence of amino acids or the derivative thereof, that are present in the larger receptor variant as identified by the method described above.

This invention provides a novel method for identifying, with efficiency and a certain degree of predictability, new physiologically active peptides. These physiologically active peptides are identified by analyzing receptors of substances associated with functional antagonism, thereby eliminating the necessity of the prior art isolation methods, physiologically active substances present in small quantities in vivo. Furthermore, because the physiological activity of the identified peptides is associated with the functional antagonism of the receptors, it may be easier to identify the physiological activities of these peptides. Thus,

this invention provides a new more efficient method for producing new physiologically active peptides.

THE BEST MODE FOR CARRYING OUT THE INVENTION

According to one method of the present invention, receptors are identified in which there is a substance or cell that is functionally antagonistic to the ligand of the receptor or a cell or substance having functional antagonism to cells which express the receptor. Functional antagonism is a fundamental process for maintaining homeostasis in the human body. There are a large number of substances or cells having mutual antagonisms within the human body. Examples of receptors in these categories are calcitonin receptors (osteoclasts on which calcitonin acts shows functional antagonism to osteoblasts), glucagon receptors (glucagon having functional antagonism to insulin), somatostatin receptors (somatostatin having functional antagonism to growth hormones), and parathyroid hormones (hormones having functional antagonism to calcitonin, etc.) and the like. Such receptors include but are not limited to 7-transmembrane type receptors such as calcitonin receptors.

The amino acid sequences or the sizes of such receptors are compared to find receptors which have one or more variants in size but are the products of the same gene. This difference in size may be a result of alternative splicing or post-translational modification. This process may be performed by identifying and comparing the sizes and amino acid sequences of identified receptors, or by making use of literature if reported therein. Some examples of receptors, wherein there are receptors having one or more variants in size while being products of the same gene are calcitonin receptors, glucagon receptors, somatostatin receptors, and the like.

After identifying receptors having one or more variants in size and products of the sane gene, the amino acid sequences of these receptors are compared with each other to identify which domain in the larger receptor is missing from the smaller receptor. The domain missing from the smaller receptor comprises the sequence of a physiologically active peptide. In other words, the peptide representing the missing domain of the smaller receptor has physiological properties. It was confirmed that, as shown in the examples below, a peptide

representing the missing domain of a larger variant of the calicitonin receptor binds to receptors present on osteoblasts thereby promoting osteogenesis.

Since the peptide representing the missing domain identified by the above-described method has physiological activities, a physiologically active substance may be obtained by producing this domain. In most cases, the missing domain comprises peptides of relatively short sizes, and therefore, in such cases, a commercially available peptide synthesizer may be used to easily produce by chemically synthesizing the peptides. Alternatively, such peptides may be produced by using a method of genetic engineering according to known methods.

The physiological activity of the peptide obtained is related to the above-mentioned functional antagonism, and therefore, the activity can be easily confirmed by any applicable suitable methods depending on each functional antagonism.

It is well-known to those skilled in the art that the physiological activity of peptides may be maintained even when some of the amino acids are substituted by other amino acids, or when some amino acids are added, or when some of the amino acids are deleted. Therefore, the present invention includes a method for producing a peptide which has physiological activities inherent to physiologically active peptides comprising the said missing domain, wherein some of the amino acids constituting the missing domain are replaced with other amino acids, or some other amino acids are added to amino acids constituting the missing domain, or some of the amino acids are deleted from the amino acids constituting the missing domain. (The foregoing substances are referred to hereinafter as "derivatives" of the missing domain.) Such a derivative preferably has not less than 70%, and more preferably 90%, of homogeneity with the above-mentioned missing domain.

EXAMPLES

The present invention is more specifically described below with reference to examples. It should be noted, however, that the present invention is not limited to the following examples.

[Example 1] Identification of the missing domain of calcitonin receptor:

The amino acid sequences of calcitonin receptors are described in Journal of Clinical Investigation, Vol. 90, No. 5 (1992). When the described amino acid sequences of calcitonin receptors described in the above reference are compared, the amino acids at 175th through 190th sequence positions of the amino acid sequence of the larger receptor are missing in the amino acid sequence of the smaller receptor. The amino acid sequence of the missing domain is shown in SEQ. ID. NO: 1.

[Example 2] Production of peptides:

By using a commercially available peptide synthesizer, a peptide having the amino acid sequence shown in SEQ. ID. NO: 1 was synthesized.

[Example 3] Proliferation promotion of osteoblasts:

ROS cells from a rat, which are osteoblasts, (available from ATCC) were cultured in F10 growth medium containing 10% fetal bovine serum (available from Dainippon Pharmaceutical), and incubated in a chamber of constant temperature of 37° C under humidified air containing 5% of CO2 gas. Using trypsin treatment, the cells were disseminated into a 24-well culture plate at the rate of 1 x 10^5 cells/well, and when the colonies became confluent, the medium was replaced with F10 medium containing 1% fetal bovine serum and the cells were cultured for 24 hours. The peptide produced in Example 2 was dissolved in F10 medium containing 1% fetal bovine serum, was added to the wells at varying quantities, and the culturing was continued for additional 24 hours.

The effect of the peptide on cell growth and poliferation was measured using the MTT assay. The assay determined the ability of the peptide to promote cell growth and

poliferation as compared to the samples without peptide treatment. The MTT assay and the calculation of growth promotion ratio were performed as follows: according to the protocol of MTT-Cell-Growth Assay kit commercially available from Funakoshi, Co., Ltd., the substance of the present invention was added to the wells at varying quantities and was left for one day and night. The number of living cells, was counted using colorimetry, making use of the phenomena of cleavage of MTT (3-4,5 dimethylthizaol-2YL)-2,5 diphenyl tetrazolium bromide to dark blue formazan by enzymes present in the mitochondria of a living cell. The following results in colorimetric value were obtained by adding varying quantities of the peptide of the present invention, while the value for the control group to which no peptide is added being at 100%. The results are shown in Table 1, below.

Table 1:

Peptide added (μg/well)	Rate of growth promotion (%)
0	100.0
0.001	109.6
0.01	110.5
0.1	636.2
1.0	1317.1

As can be seen from Table 1, peptide identified according to the method of the present invention promoted the growth and poliferation of osteoblasts. Thus the peptide of the present invention is thought to cause a increase in bone density, and thus may be useful in the treatment of osteopathy, such as osteoporosis and the like.

[Example 3] Presence on osteoblasts of the receptors of the peptides of the present invention:

The peptide of the present invention was found to promote the growth of osteoblasts. It was estimated that osteoblasts have receptors for the peptides of the present invention. If the receptors are present, the peptide of the present invention could possibly be a peptide which is found naturally in vivo. Therefore, it was investigated whether the osteoblasts have receptors for the peptides.

The peptide obtained in Example 2 was labeled with biotin, and a predetermined quantity was added to ROS cells cultured in a similar method as in Example 3. Unlabelled peptide dissolved in F10 medium containing 10% fetal bovine serum was then added in varying quantities to effectuate a competitive reaction. The foregoing experimental operation was performed more specifically as follows: the peptide of the present invention was biotinated according to the protocol of Protein Biotin-Labeling kit from SUMILON Co., and a predetermined quantity of the biotinated peptide was added to a predetermined number of cells disseminated in wells, followed by adding 0-0.512 μg/well of non-labeled peptide to each well to effectuate a competitive reaction for 6 hours, and thereafter, the cells were rinsed with PBS and the biotinated peptides bound to receptors on the cell surface were reacted with peroxidase labeled by streptavidin to observe a color development reaction. When any receptor of the peptides of the present invention is present on the cell surface, a competitive reaction with the un-labeled peptides is effected and the color intensity is decreased. The results are shown in Table 2 below:

Table 2:

Added amount of Peptides of Non-labeled type (μ/g/well)	Ratio to added amount of peptides of labeled type (%)
0	100
0.032	98.4
0.064	86.5
0.128	79.6
0.256	34.1
0.512	29.5

As shown in Table 2, the ratio to the added amount of the peptides of labeled type varies depending on the amount of non-labeled peptides added, and therefore, proving that the osteoblasts have receptors for the peptide of the present invention. Therefore, it is implied that the peptide of the present invention may be a peptide which occurs naturally in vivo.

[acute toxicity test]

An acute toxicity test of the peptide produced in Example 2 was performed by using ddy male mice (weight 40-45 grams). The peptide of the present invention was dissolved into a saline solution (pH 6.0) and administered to the mice through the caudal vein. Thereafter, the mice were subjected to observation for 14 days. The dosage was set at 1, 10, and 100 μg/kg. The results are shown in Table 3 below:

Table 3

Dosage (μg/kg)	Mortality
1	0/5
10	0/5
100	0/5

[Example 4] Identification of the missing domain in glucagon receptors:

Using the same methodology as in Example 1, the amino acid sequences of glucagon receptors described in FEBS Letters 351 (1994), pp. 271-275, were compared to identify the amino acid sequence of the domain being present in the a larger receptors but missing in the smaller receptors of smaller size. The amino acid sequence of the missing domain thus identified is shown in Sequence 2.

[Example 6] Identification of the missing domain in somatostatin receptors:

Using the same methodology as in Example 1, the amino acid sequences of somatostatin receptors described in Molecular Pharmacology, 44: pp. 1008-1015 (1993), were compared to identify the amino acid sequence in the domain present in the larger receptors but missing in the smaller receptors. The amino acid sequence of the missing domain thus identified is shown in Sequence 3.

SEQUENCE LISTING

SEQ ID NO: 1

SEQUENCE LENGTH:

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

SEQUENCE DESCRIPTION:

Lys Leu Thr Thr Ile Phe Pro Leu Asn Trp Lys Tyr Arg Lys Ala Leu
1 5 10 15

SEQ ID NO: 2

SEQUENCE LENGTH:

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

SEQUENCE DESCRIPTION:

Gly Asn Gly Val Val Ser Ala Trp Glu Ala Glu Gly Ala Lys Ser Gly
1 5 10 15
Ser Gly Leu Thr Arg Ala Tyr Thr His Val Pro
20 25

SEQ ID NO: 3

SEQUENCE LENGTH:

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

SEQUENCE DESCRIPTION:

Pro Ser Cys Gln Trp Val Gln Ala Pro Ala Cys Gln
1 5 10

This substitute specification is submitted, in response to a requirement by the Examiner.

Accompanying this transmittal is a statement, as required by 37 C.F.R. § 1.125, that the substitute specification transmitted herewith contains no new matter.

Respectfully submitted,

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